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SHORT REPORTS

Expression and regulation of Cyr61 in human breast cancer cell lines

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We have shown that Cyr61, an angiogenic regulator, is overexpressed in invasive and metastatic human breast cancer cells and tumor biopsies. We have further demonstrated that Cyr61 promotes acquisition of estrogen-independence and anti-estrogen resistance *in vivo* in breast cancer cells. Moreover, we have demonstrated that Cyr61 induces tumor formation and tumor vascularization *in vivo*, events mediated through the activation of the MAPK and the Akt signaling pathways. Here we investigate how Cyr61 expression is regulated in both estrogen receptor (ER)-positive and ER-negative breast cancer cells. We demonstrate that Cyr61 mRNA and protein expression is inducible by estrogen and anti-estrogens in ER-positive breast cancer cells. We show that a labile protein as well as a negative regulator might be involved in Cyr61 expression in estrogen-dependent breast cancer cells. Other important regulators of Cyr61 expression in breast cancer cells that we found are the phorbol ester TPA, vitamin D, and retinoic acid. TPA causes positive regulation of Cyr61 expression in ER-positive MCF-7 cells. Vitamin D induces a transient stimulatory effect on Cyr61 gene expression. Lastly, retinoic acid has a negative effect on Cyr61 expression and downregulates its expression in MCF-7 cells. Interestingly, most of these effects are not seen in aggressive breast cancer cells that do not express ER and express high levels of Cyr61, such as the MDA-MB-231 cells. Our results are in agreement with our knowledge that Cyr61 promotes tumor growth, and that tumor-promoting agents have a positive impact on cells that express low levels of Cyr61, such as the ER-positive breast cancer cells; however, these agents have no significant effect on cells that express high levels of Cyr61. Our findings suggest an association between increased Cyr61 expression and an aggressive phenotype of breast cancer cells.
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We have previously identified an angiogenic regulator, Cyr61, as a protein potentially involved in breast cancer progression (Tsai *et al*., 2000). Cyr61 belongs to the CCN family, which consists of Cyr61, CTFG, Nov, WISP-1, WISP-2, and WISP-3 (Lau and Lam, 1999). These structurally conserved proteins share four modular domains with similar sequence homologies (Lau and Lam, 1999). Cyr61 is a cysteine-rich, heparin-binding protein that is secreted and associated with the cell surface and the extracellular matrix (ECM) (Yang and Lau, 1991; Kirreva *et al*., 1997). Cyr61 mediates cell adhesion, induces cellular migration, enhances growth factor-induced DNA synthesis in fibroblasts and endothelial cells, stimulates chemotaxis of fibroblasts and endothelial cells, and increases chondrogenesis in mesenchymal cells (O'Brien and Lau, 1992; Kireeva *et al*., 1996, 1997, 1998; Frazier *et al*., 1996; Wong *et al*., 1997; Kolesnikova and Lau, 1998; Babic *et al*., 1998), possibly through its binding to integrin receptors, such as $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha IIb\beta 3$, and $\alpha 6\beta 1$ (Kireeva *et al*., 1998; Jedsadayanmata *et al*., 1999; Chen *et al*., 2000; Grzeszkiewicz *et al*., 2001). Most significantly, expression of Cyr61 enhances neovascularization and tumor formation of human tumor cells in immunodeficient mice (Babic *et al*., 1998, 1999; Xie *et al*., 2001).

We have shown that Cyr61 is overexpressed in invasive and metastatic human breast cancer cells and tumor biopsies (Tsai *et al*., 2000). In addition, we demonstrated that Cyr61 function is necessary for heregulin (HRG)-mediated chemomigration of breast cancer cells (Tsai *et al*., 2000). We further determined that Cyr61 is sufficient to promote breast cancer cells to bypass their normal estrogen requirement for growth, to induce progression of breast cancer cells to more aggressive and invasive phentoypes *in vitro*, and to induce tumor formation *in vivo* (Tsai *et al*., 2001, manuscript submitted). To understand better the mechanism of Cyr61 action in promoting breast cancer progression, it is important to investigate how Cyr61 expression is regulated in breast cancer cells.

It is well known that Cyr61, an immediate-early gene, is transcriptionally activated by serum, platelet-derived growth factor (PDGF), transforming growth factor-$\beta 1$ (TGF-$\beta 1$), and basic fibroblast growth factor (bFGF) in fibroblasts and neuronal cells (Lau and Nathans, 1987; O'Brien *et al*., 1992; Ryseck *et al*., 1991; Bunner *et al*., 1991). Other cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-

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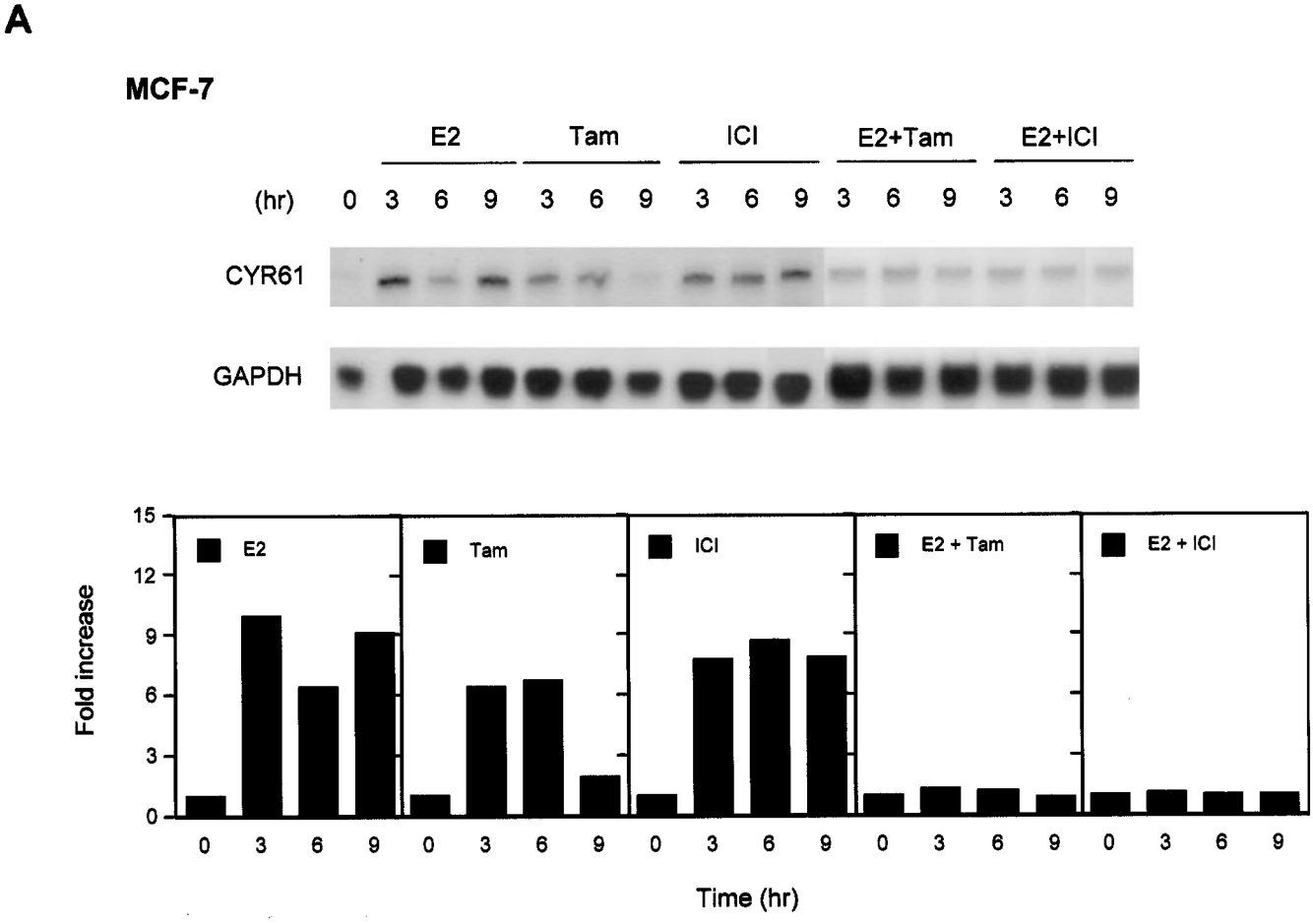
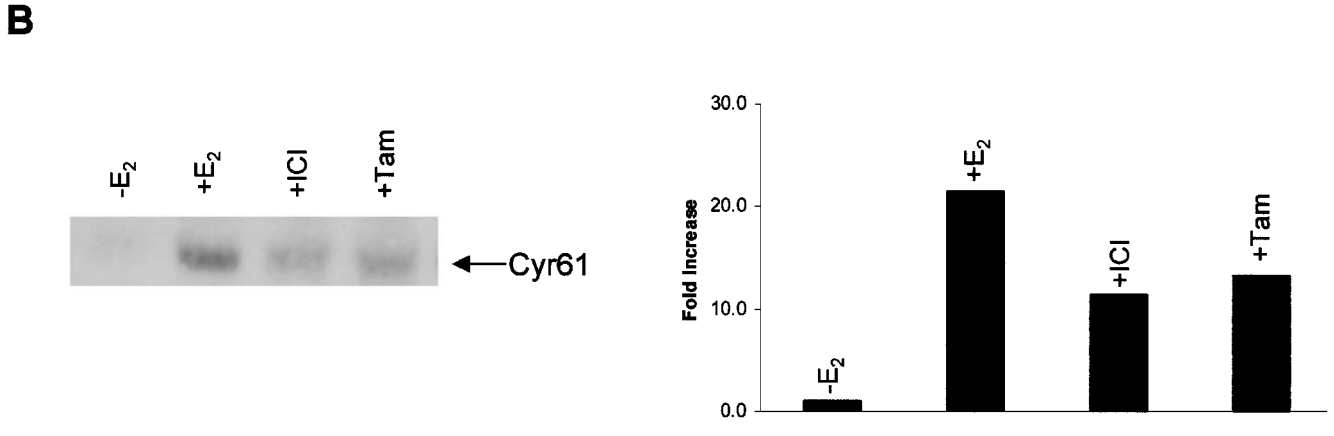
1) also strongly elevate the expression of Cyr61 (Scheutze *et al*., 1998). Cyr61 is differentially expressed by muscarinic acetylcholine receptors (mAChRs) in the brain (Albrecht *et al*., 2000), and by factor VIIa and thrombin in human fibroblasts (Pendurthi *et al*., 2000). Phorbol ester 12-*O*-tetradecanoyphorbol-13-acetate (TPA) mediates Cyr61 expression in the liver (Nathans *et al*., 1988). Incidentally, others and we have found expression of Cyr61 involving protein kinase C (PKC) and the MEK/MAPK pathways (Chung and Ahn, 1998; Inuzuka *et al*., 1999; Albrecht *et al*., 2000; Tsai *et al*., 2001, manuscript submitted). Cyr61 is also vitamin D-responsive in human osteoblasts and osteosarcoma cell lines (Scheutze *et al*., 1998). Induction of Cyr61 may be attenuated by glucocorticoids in murine fibroblasts (Smith and Herschman, 1995). Moreover, previous studies showed that murine Cyr61 is inducible by estrogen (E2) and tamoxifen (Tam) in the uterus of ovariectomized rats (Rivera-Gonzalez *et al*., 1998). However, it is not clear how Cyr61 is regulated in human breast cancer, except it has been indicated that Cyr61 is E2-inducible, and that Tam inhibited its





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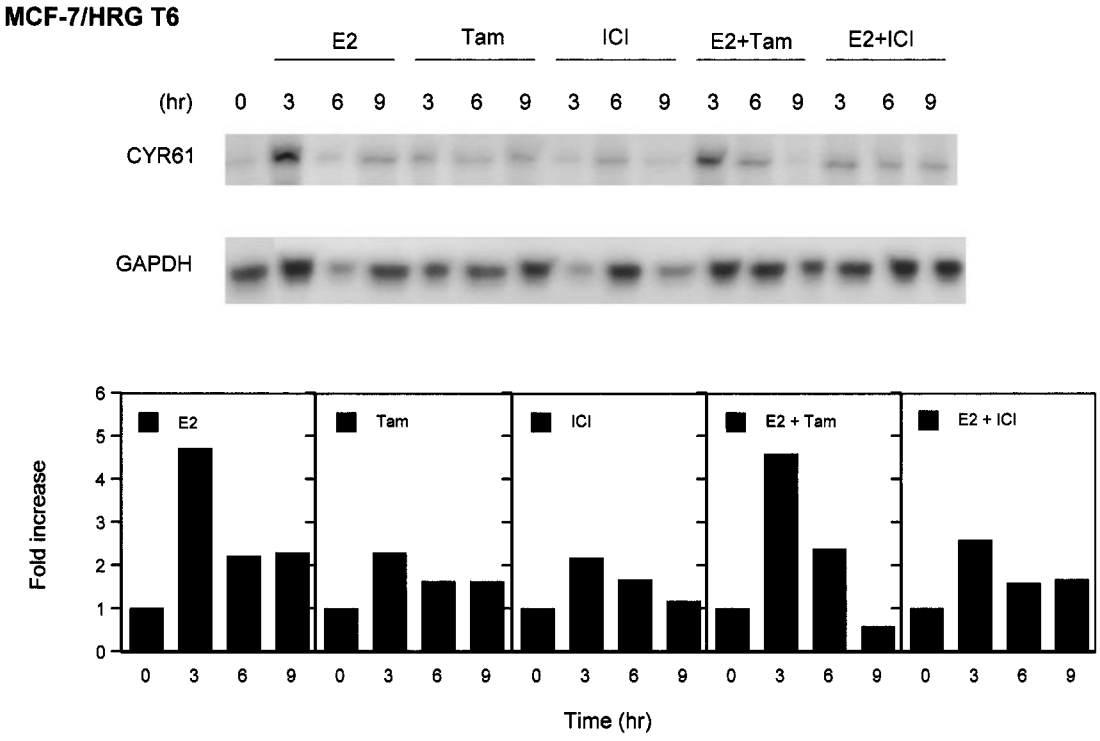
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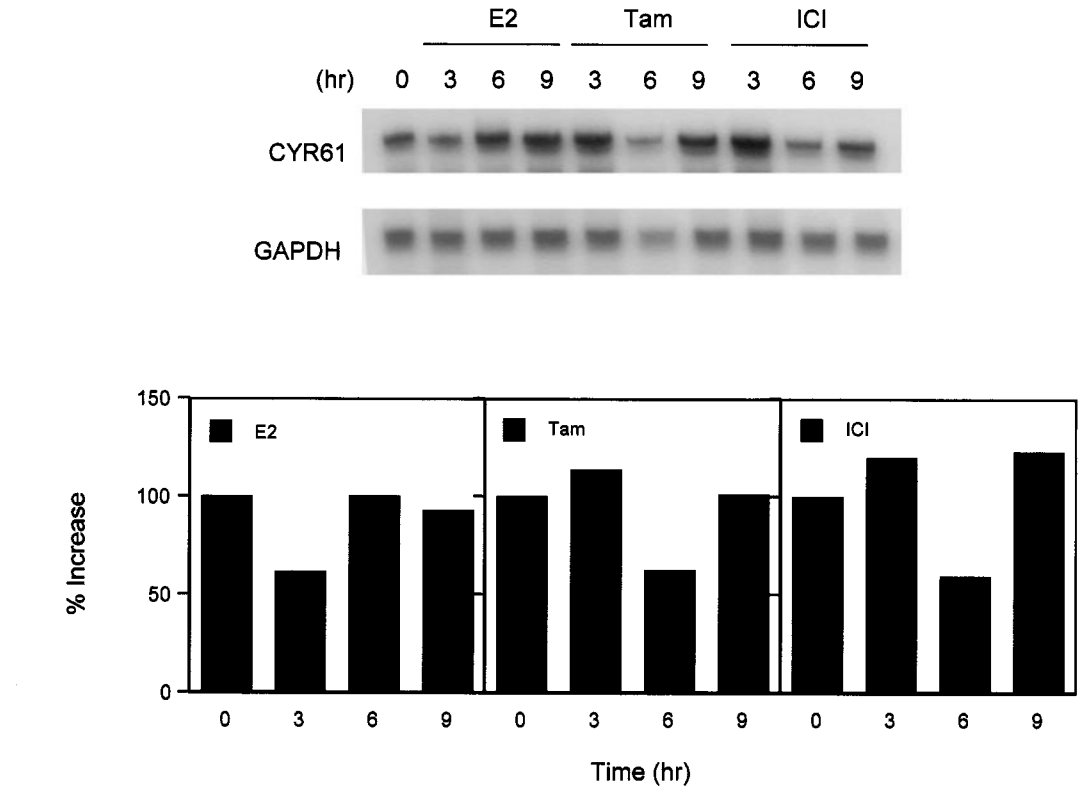
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expression in human breast cancer cells (Xie *et al*., 2001).

We have recently shown that Cyr61 is expressed in breast cancer cells, and that enhanced expression of Cyr61 correlates with lack of ER expression and with advanced breast cancer diseases (Tsai *et al*., 2000). Here we investigate in more detail how Cyr61 expression is regulated in both ER-positive and ER-negative breast cancer cells. To determine whether Cyr61 expression was regulated by estrogen, anti-estrogens (anti-E2), or both, in breast cancer cells that express or do not express ER, the levels of Cyr61 mRNA were measured in a time-dependent manner by RNase protection assays. Cells were grown in phenol red-free media containing FBS depleted of estrogenic compounds by charcoal adsorption, and treated in the presence or absence of E2.

As shown in Figure 1a, the mRNA levels of Cyr61 in MCF-7 cells under the condition of estrogen depletion was very low or nearly undetectable. In contrast, E2 markedly increased the expression of Cyr61 mRNA. Upregulation of Cyr61 was observed as early as 3 h after E2 treatment, as a 6- to 10-fold increase (Figure 1a, top panel). The Cyr61 transcript, although slightly reduced after 9 h of treatment, was sustained for up to 72 h without significant decreases (data not shown).

To provide a better understanding of the possible mechanism of anti-estrogenic effects, two distinct anti-estrogens, Tam and ICI 182, 780 (ICI), were used. Tam functions as an antagonist as well as a partial agonist by binding to the ligand-binding domain, thus blocking transactivation of the AF-2 domain but not the AF-1 of the ER. ICI, a pure anti-estrogen, displays solely antagonistic effects by binding to the ER, disrupting

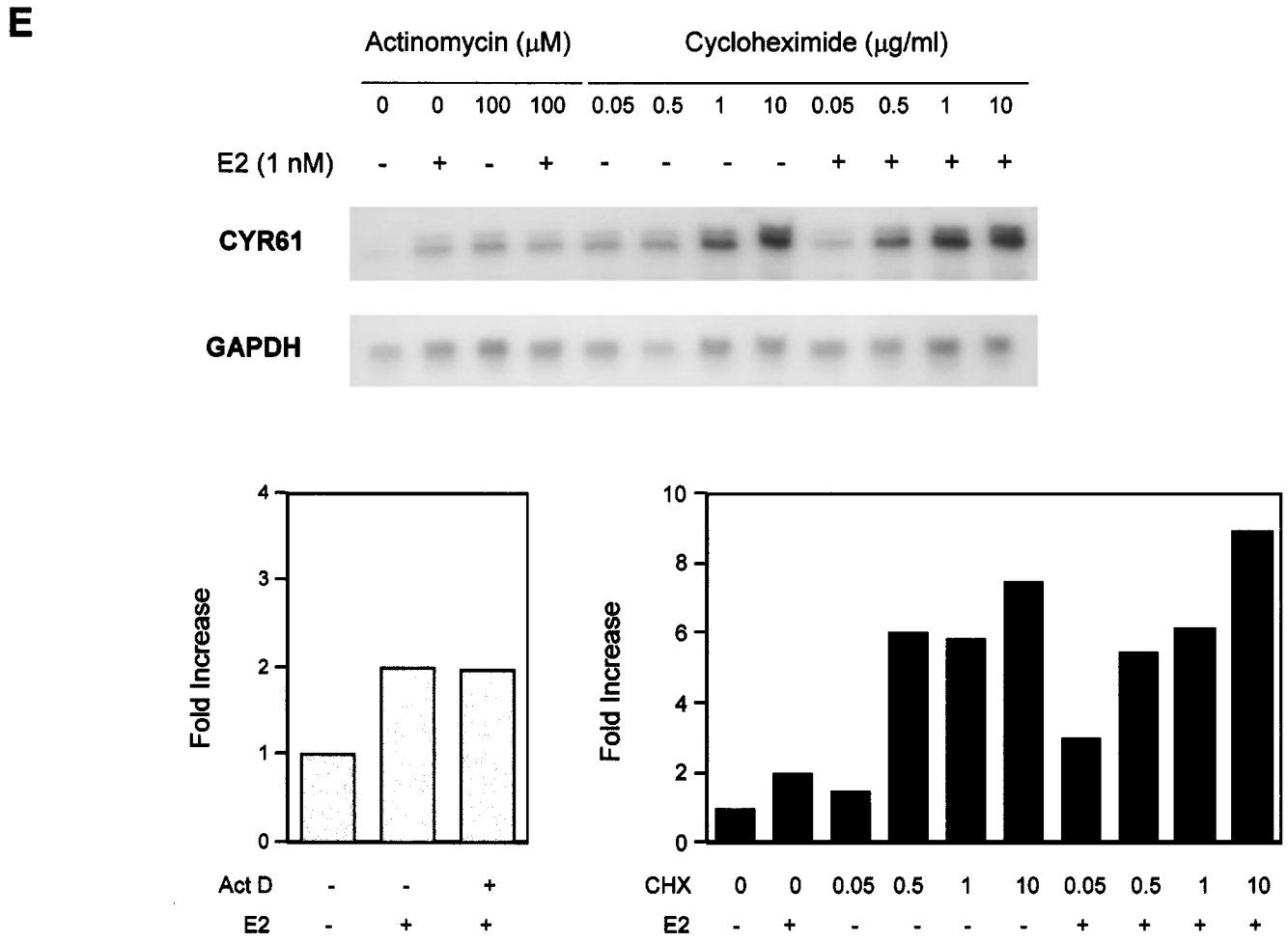
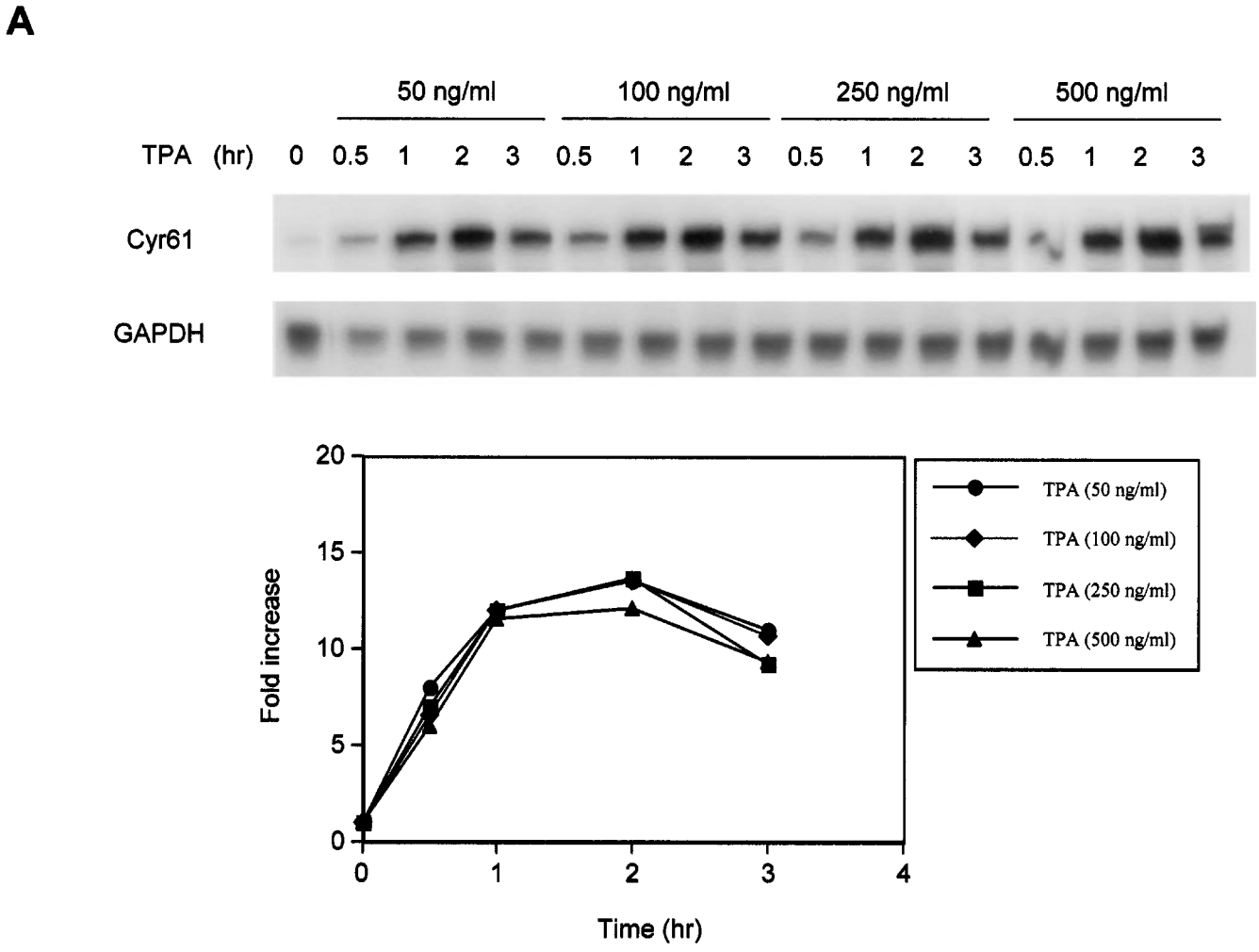


Figure 1 Effect of estrogen, anti-estrogen, actinomycin D, and cycloheximide on Cyr61 expression in breast cancer cells. Breast cancer cells, (**a**) MCF-y, (**b**) MCF-7, (**c**) MCF-7/HRG clone T6, and (**d**) MDA-MB-231, were cultured in E2-depleted media for 4 days. For (**a**,**c**,**d**), total RNA was isolated at 0, 3, 6 and 9 h after treatments with E2 (1 nM), Tam (100 nM), ICI (100 nM), the combination of E2 and Tam, and the recombinant of E2 and ICI. The RNA was analysed for Cyr61 expression by the RNase protection assay as described previously (Tsai *et al*., 2000). For (**b**), conditioned media was collected after 24 h of E2, Tam or ICI treatment and concentrated 50×. Western blot analysis was then performed using 20 μl of the concentrated media. (**e**) MCF-7 cells were cultured in E2-depleted media and incubated with a control vehicle, and with E2 (1 mM) in the presence or absence of actinomycin D (Act-D; 100 nM) or cycloheximide (CHX; 0.05, 0.5, 1, and 10 μg/ml) for 6 h. Total RNA was isolated and analysed by the RNase protection assays as above. GAPDH is used as a control of RNA loading. Data were quantified by densitometry and normalized to the level of GAPDH. Similar results were obtained from at least four independent experiments



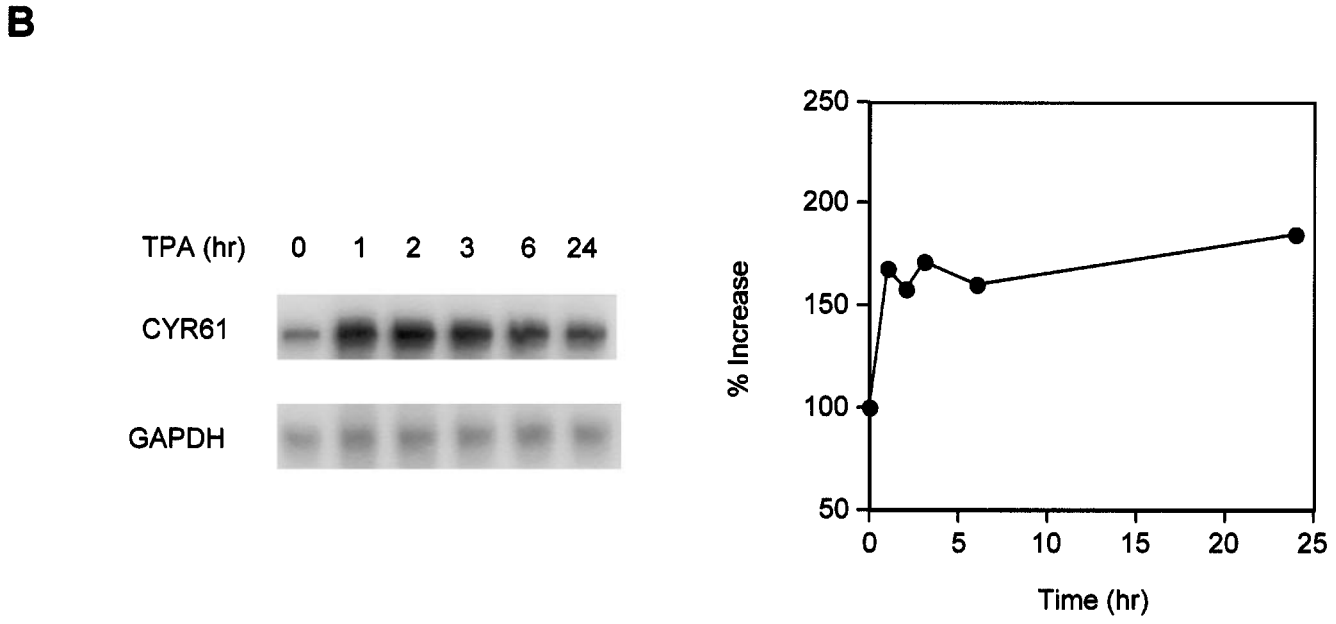


Figure 2 Effect of TPA on Cyr61 mRNA expression in breast cancer cells. (**a**) MCF-7 cells were treated with TPA (50, 100, 250, and 500 ng/ml) for 0, 0.5, 1, 2, and 3 h. Total RNA was isolated and analysed by the RNase protection assay. (**b**) MDA-MB-231 cells were treated with 50 ng/ml TPA for 0, 1, 2, 3, 6, and 24 h. Total RNA was isolated and analysed as described above. Data were quantified by densitometry and normalized to the level of GAPDH. Similar results were obtained from at least three independent experiments

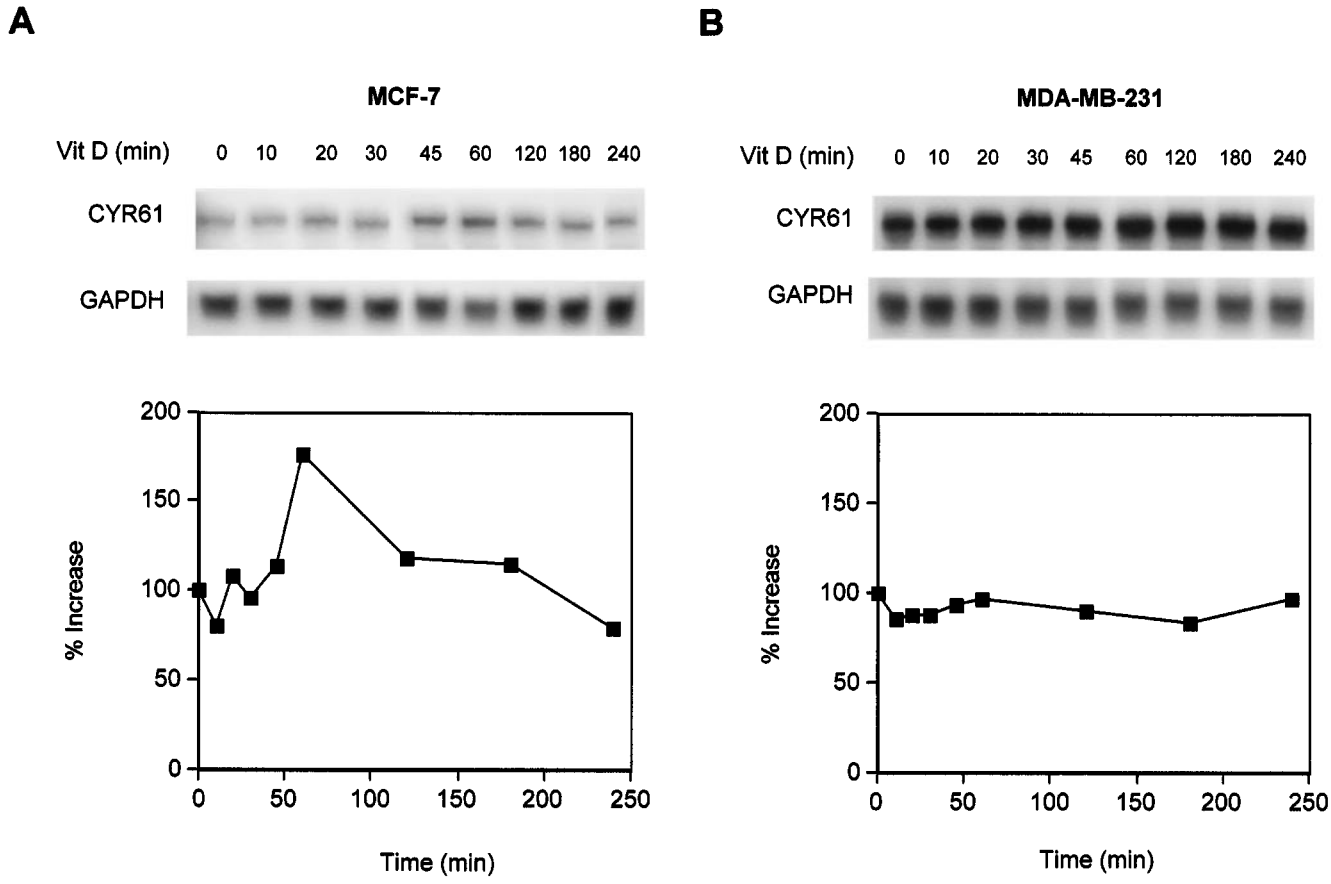


Figure 3 Effect of vitamin D on Cyr61 mRNA expression in breast cancer cells. (**a**) MCF-7 cells and (**b**) MDA-MB-231 cells were treated with vitamin D (10 mM) for 0, 10, 20, 30, 45, 60, 120, 180, and 240 min. Total RNA was isolated and analysed for Cyr61 mRNA levels by the RNase protection assays. Data were quantified by densitometry and normalized to the level of GAPDH. Similar results were obtained from at least three independent experiments

the nuclear translocation of the ER, and inducing rapid degradation of the ER (MacGregor and Jordan, 1988). We examined the effects of the anti-estrogens on E2 induction of Cyr61 expression. Cells were treated with E2 alone, or with the combination of E2 and anti-E2. Both anti-estrogens exerted antagonistic effects on E2.

Interestingly, treatment with either anti-estrogen on MCF-7 cells caused a marked upregulation of Cyr61 mRNA (Figure 1a, top panel). Tam induced the expression of Cyr61 mRNA by a sixfold increase after 3 h of treatment, and then its expression was reduced to the basal level after 9 h. On the other hand, ICI, a pure E2 antagonist, induced rapid Cyr61 upregulation kinetics (Figure 1a, top panel), similar to that observed for E2; that is, the accumulated Cyr61 mRNA was seen as early as 3 h after treatment and maintained for up to 72 h (data not shown). Interestingly, the combination of E2 with Tam or ICI abrogated the expression of Cyr61. This effect can be due to a change in receptor occupancy and the modification of the ER pockets that each of these compounds interfere with. Quantification of Cyr61 protein expression was performed using densitometry (Figure 1a, bottom panel).

Moreover, we demonstrated that Cyr61 protein expression was also upregulated (over 10–20-fold) by E2, Tam and ICI in MCF-7 cells (Figure 1b). Quantification of the Cyr61 expression was performed using densitometry. These cells were grown in phenol red-free media containing FBS depleted of estrogenic compounds by charcoal adsorption for 4 days and treated with 10^{-9} M E2 for 24 h. The media was then collected and concentrated 50 times. Western blot analysis was performed as previously described (Tsai *et al*., 2000). While writing this manuscript, these data were confirmed recently by Sampath *et al*., 2001.

We then examined the expression of Cyr61 in E2-independent and anti-E2-resistant breast cancer cells, such as HRG-transfected MCF-7 clones (MCF-7/HRG) (Tang *et al*., 1996) and MDA-MB-231. We first determined whether E2-induced Cyr61 expression is mediated in one of the MCF-7/HRG clones (clone T6), all of which express high levels of HRG and moderate levels of Cyr61. Both E2 and anti-E2 transiently induced Cyr61 expression in MCF-7/HRG cells. The highest expression was observed after 3 h of E2 treatment, returning to the basal level shortly after 9 h of treatment. Although the MCF-7/HRG cells acquired an E2-independent phenotype both *in vitro* and *in vivo* (Tang *et al*., 1996), it appears that these cells respond slightly to E2 in regulating Cyr61

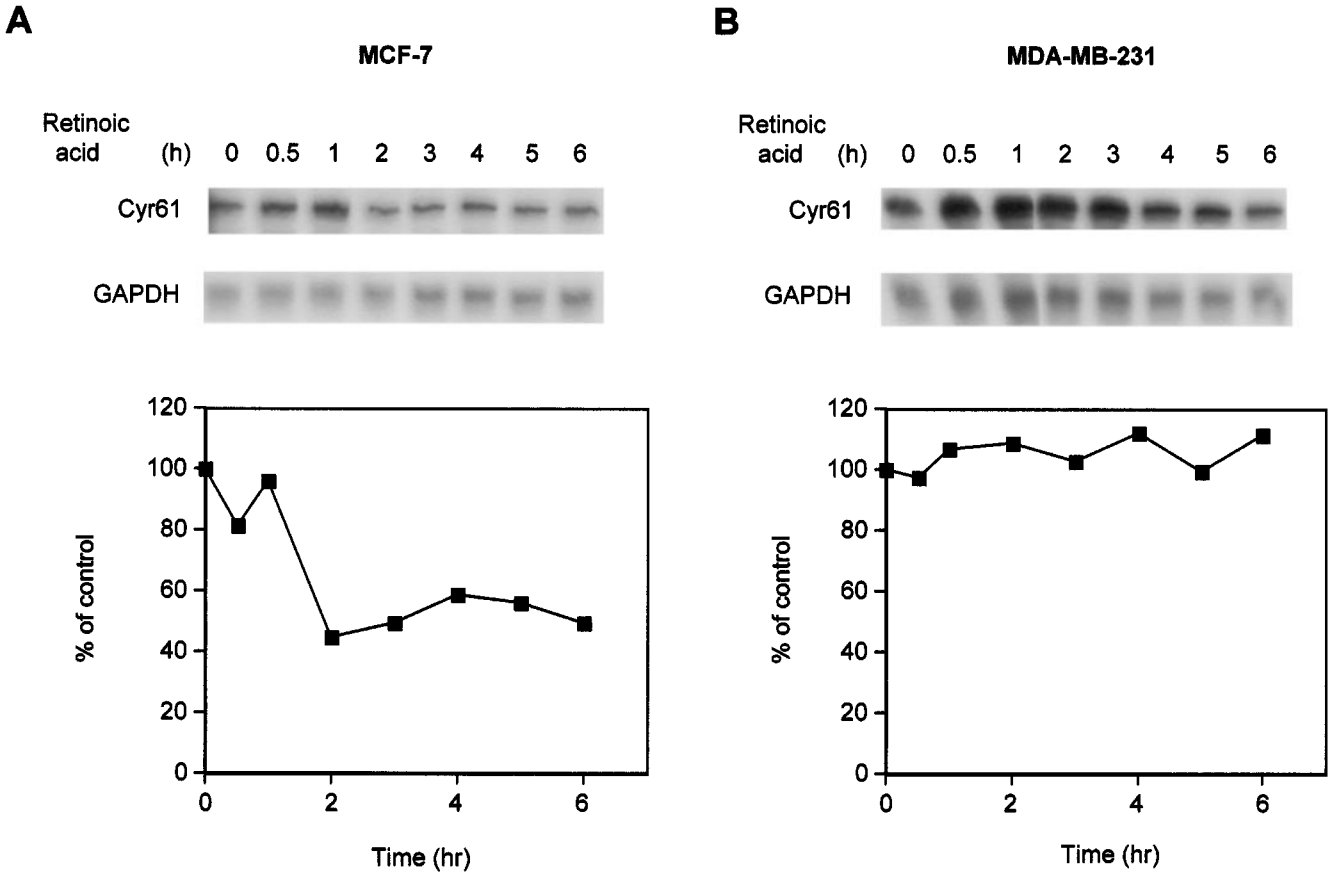


Figure 4 Effect of retinoic acid on Cyr61 mRNA expression in breast cancer cells. (**a**) MCR-7 cells and (**b**) MDA-MB-231 cells were treated with 10 nM all trans-retinoic acid for 0, 0.5, 1, 2, 3, 4, 5, and 6 h. Total RNA was isolated and analysed for Cyr61 mRNA levels by the RNase protection assay. Data were quantified by densitometry and normalized to the level of GAPDH. Similar results were obtained from at least three independent experiments

expression, however with different kinetics as compared to wild-type MCF-7 cells (Figure 1c). Furthermore, the level of Cyr61 induction by E2 and anti-E2 in the MCF-7/HRg cells was 2–3-fold lower as compared to wild-type MCF-7 cells. Moreover, while ICI blocked E2-mediated Cyr61 expression by 50 to 70% as early as 3 h after treatment, Tam showed an apparent delayed blockage of 50% after 6 h of treatment and a complete blockage after 9 h of treatment (Figure 1c). Thus, the effects of Tam and ICI on E2 induction of Cyr61 expression appear to result from distinct pathways in MCF-7/HRG cells, possibly because these cells become resistant to both Tam and ICI, while only Tam has agonistic effect and induces tumor growth *in vivo* (Atlas *et al*., 2001, manuscript submitted). Quantification of the Cyr61 expression is shown in the bottom panel of Figure 1c using densitometry.

Next we examined the effect of E2 on Cyr61 expression in breast cancer cells that do not express ER. As expected and shown in Figure 1d, E2 and anti-E2 no longer mediate the expression of Cyr61 in MDA-MB-231 cells, which express high levels of endogenous Cyr61. This finding is consistent with the phenotypes of MDA-MB-231, which is ER-negative, E2-independent, anti-E2-resistant, highly invasive, and metastatic *in vivo*. Quantification of the Cyr61 expression was performed using densitometry and is shown in Figure 1d, bottom panel.

Together, our results indicate that the fold induction of Cyr61 by E2 and or anti-E2 coincides with the endogenous levels of Cyr61 expression and is inversely correlated with the levels of ER expression in breast cancer cells as shown in Figure 1a,c,d. Here we demonstrate that E2, a tumor-promoting hormone, highly induces the expression of Cyr61 in breast cancer cells that express ER. In cells that acquire E2-independence but still express ER, such as the MCF-7/HRG cells, E2 induces Cyr61 to a much lower extent. In contrast, in cells that are very aggressive, do not express ER, and express high levels of Cyr61, E2 fails to further induce Cyr61 expression. These results are consistent with the hypothesis that Cyr61 expression correlates with the aggressiveness of the breast tumors, which in turn correlates with the lack of ER expression (Tsai *et al*., 2000).

Our data clearly demonstrate that Cyr61 is an E2-inducible gene in breast cancer cells. To investigate further whether transcription is involved in the E2-mediated increase in Cyr61 mRNA steady-state levels, MCF-7 cells were depleted of E2-like compounds and

incubated with E2 (1 nM) and actinomycin-D (100 nM), which is an RNA synthesis inhibitor. As shown in Figure 1e, we demonstrate that E2 stimulates Cyr61 expression in MCF-7 cells (Figure 1e), and that actinomycin-D treatment (100 μM) has no effect on E2-mediated Cyr61 expression, indicating that *de novo* RNA synthesis is not required in E2 induction of Cyr61. The fact that actinomycin-D upregulates the mRNA levels suggests that although the *de novo* RNA synthesis is inhibited there is upregulation of Cyr61. If so, *de novo* of Cyr61-mRNA cannot account for that. It is therefore possible that the upregulation is due to the increased stability of the RNA, and the inability of protein(s) that may be involved in the recycling of the mRNA in question are not transcribed. This is supported by the fact that when protein synthesis is inhibited there is also upregulation of mRNA, supporting the same possible mechanism, or in the later case the relief of repression. It is also possible that E2 increases the stability of the mRNA. We then determined whether *de novo* protein synthesis is necessary for the induction of Cyr61 by E2. MCF-7 cells were depleted of E2-like compounds prior to the treatment with E2 in the presence or absence of a protein synthesis inhibitor, cycloheximide. As shown in Figure 1e, the stimulatory effect of E2 was not blocked by cycloheximide. Interestingly, cycloheximide treatments markedly increased the basal level of Cyr61 expression and further increased E2-induced Cyr61 mRNA accumulation by 6–7-fold in a dose-dependent manner. These results indicate that a rapid accumulation of Cyr61 mRNA involved a labile protein(s), and that cycloheximide prevented the *de novo* protein synthesis of a negative regulator of Cyr61. Our results are consistent with the data by Sampath *et al*., 2001 that cycloheximide does not block E2-regulated Cyr61 expression. However, we showed that cycloheximide alone regulated Cyr61 expression. In addition, our results clearly demonstrate the increase in Cyr61 expression in the presence of cycloheximide at three different concentrations in the presence or absence of physiological concentration of E2 (1 nM), whereas their data have only one concentration of cycloheximide and a 10 times higher concentration of E2 (10 nM). The discrepancy between our findings and Sampath *et al*. (2001) could be due to the known clonogenicity of MCF-7 cells. In addition, studies with actinomycin-D or cycloheximide alone were not provided in the above-cited publication. We have followed up the E2 effect on Cyr61 expression, and the Cyr61 mRNA level was sustained up to 72 h without significant decrease.

It is known that nuclear steroid/thyroid/retinoid receptors and c-*erbB* membrane receptor tyrosine kinases control epithelial cell growth and differentiation. Retinoic acid receptor (RAR) can dimerize with the vitamin D receptor (VDR), the glucocorticoid receptor, or the thyroid receptor (Schneider *et al*., 1999). Molecular interactions between RARs and c-*erbB*2, ER and c-*erbB*2, and between ER and RARα have been reported (Flicker *et al*., 1997; Schneider *et al*., 1999). Moreover, these nuclear receptors and protein kinase signaling, such as protein kinase A (PKA) and protein kinase C (PKC), communicate with each other, perhaps via cross-talk regulatory factors (Schneider *et al*., 1999). It is well documented that retinoid signaling via retinoic acid (RA) and retinoid X receptors (RARs and RXRs) regulates mammary epithelial cell growth, differentiation, morphogenesis, and apoptosis (Glass *et al*., 1997). Therefore, we next investigated whether TPA, vitamin D, and retinoic acid (RA) play any role in the regulation of Cyr61 expression in breast cancer cells.

TPA is an effective regulator of growth of many different cell types. It activates PKC, which plays a key role in the control of many signaling pathways involved in growth, differentiation, and cellular transformation. Previous studies have shown that Cyr61 is induced by phorbol ester TPA in fibroblasts (Nathans *et al*., 1988). It has been suggested that Cyr61 may involve PKC pathways in preadipocyte cells (Inuzuka *et al*., 1999). Since activation of the PKC pathways has been shown to be a possible mechanism in the acquisition of drug resistance and of aggressive behavior in breast cancer cells (Bowden *et al*., 1999), we tested the effects of TPA on the expression of Cyr61 in breast cancer cells. As shown in Figure 2a, TPA induces upregulation of Cyr61 expression (mRNA) in MCF-7 cells in a time-dependent manner, in any dose used (from 50 ng/ml to 500 ng/ml). Cyr61 transcripts were seen to increase rapidly after 30 min of treatment with TPA, reached the peak level after 2 h of treatment (a 15-fold increase), and were maintained at that high level longer than 24 h (data not shown). Interestingly, TPA induced the expression of Cyr61 expression in MDA-MB-231 cells by about 1.5-fold and this is sustained up to 24 h (Figure 2b). These results indicate that activation of the PKC pathways may be involved in the upregulation of Cyr61, resulting in the progression of breast cancer cells.

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 (1,25-$(OH)_{2D3}$ and its several analogues are novel putative anticancer agents, with their ability to induce growth inhibition, differentiation, and apoptosis in tumor cells. 1,25-$OH)_{2D3}$ binds to the nuclear VDR with high affinity and elicits its action regulating gene expression in target cells by binding to vitamin D-responsive elements (Haussler *et al*., 1995). Cyr61 mRNA steady-state levels have been shown to be stimulated by 1,25-$(OH)_{2D3}$ in osteoblasts (Scheutze *et al*., 1998). Moreover, a close correlation between VDR abundance and cell proliferation rate has been shown in MCF-7 breast cancer cells, HL-60 myeloblastic cells, and several leukemia cell lines (Folgueira *et al*., 2000). Thus, we investigated whether Cyr61 expression in breast cancer cells could also be mediated by 1,25-$(OH)_{2D3}$. As shown in Figure 3a, 1,25-$(OH)_{2D3}$ exerts rapid and transient induction of Cyr61 steady-state mRNA levels in MCF-7 cells. In these cells, Cyr61 expression was upregulated by 1,25-$(OH)_{2D3}$ after 1 h of treatment, an effect that rapidly declined to basal levels after 2 h of treatment. By contrast, 1,25-$(OH)_{2D3}$ has no effect on Cyr61 expression in MDA-

MD-231 cells (Figure 3b). It is possible that the level of the VDR is different between ER-positive and ER-negative breast cancer cells (Agadir *et al*., 1999; Escaleira and Brentani, 1999). More likely, the extent of transcriptional co-activators and/or co-repressors, and the ligand availability of heterologous steroid hormone receptors, such as RAR and RXR, may determine the diverse function of VDR in breast cancer cells. In addition, 1,25-$(OH)_{2D3}$ has been shown to downregulate ER in MCF-7 cells (Nolan *et al*., 1998), which may cause the transient, low-level of induction of Cyr61 by 1,25-$(OH)_{2D3}$. Our results indicate that inhibitory pathways of vitamin D may involve regulation and participation of certain growth factors, such as Cyr61. The inhibitory pathways of Vitamin D may involve regulation and participation of growth factors, such as Cyr61. Since Vitamin D transiently regulates the expression of Cyr61 in MCF-7 cells (Figure 3a) and does not regulate its expression in MDA-MB-231 cells (Figure 3b), it is conceivable that Cry61 plays little or no role, in the development of Vitamin D resistance. Since all trans-retinoic acid is a potent regulator of growth of cancer cells, and RA exerts a growth inhibitory effect on ER-positive but not on ER-negative breast cancer cells (Sheikh *et al*., 1994; Srivastava *et al*., 1999), and since Cyr61-over-expressing breast cancer cells acquire a growth advantage, we studied whether Cyr61 was an RA-targeted gene in MCF-7 cells. MCF-7 cells were treated with tRA in a time- and dose-dependent manner. Interestingly, Cyr61 expression was rapidly down-regulated by RA in MCF-7 cells. This inhibitory effect was evident at 2 h, at which time Cyr61 expression was decreased to 40–45%, as compared to the control (Figure 4a). On the other hand, RA has no effect on the Cyr61 level in MDA-MB-231 cells (Figure 4b). These results are consistent with the notion that RA exerts growth-inhibitory effects on breast cancer cells by ER-mediated enhancement of RAR levels, and possibly via an ER-dependent downregulation of growth-promoting factor(s).

In summary, we have demonstrated that Cyr61 is regulated by E2, and that anti-estrogens can block this induction in MCF-7 cells. Interestingly, we determined that a possible negative regulator of Cyr61 is present in MCF-7 cells. By contrast, the effects are different when Cyr61-overexpressing cells are treated with these agents. In addition, we demonstrated that both TPA and vitamin D upregulate the expression of Cyr61 in breast cancer cells that express high levels of ER, such as MCF-7 cells. In contrast, the differentiating agent RA inhibits the expression of Cyr61 in MCF-7 cells. All of these results are consistent with our previous findings that Cyr61 promotes breast cancer cells and tumor growth, and that tumor-promoting agents have a positive impact on cells in which Cyr61 expression is low, whereas these agents have no significant effect on cells that express high levels of Cyr61. On the other hand, downregulation of the growth-promoting activity of Cyr61 by RA may account for the ability of RA to induce growth inhibition and apoptosis in breast cancer cells.

In conclusion, we demonstrate that regulation of Cyr61 is involved in breast cancer progression, and that Cyr61 should be considered as a new target for therapy. We have demonstrated that Cyr61 is highly expressed in breast carcinomas and that it induces the progression of breast cancer *in vitro* and *in vivo*. We are therefore currently investigating the mechanisms that account for Cyr61 induction of breast cancer progression.

Acknowledgments

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